Reprinted from
JOURNAL OF APICULTURAL RESEARCH
A journal of the Bee Research Association

CATALASE IN HONEY

ABNER I. SCHEPARTZ & MARY H. SUBERS

Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, 600 East Mermaid Lane, Philadelphia, Pennsylvania 19118, *U.S.A.*

Manuscript received for publication 14th January 1966

SUMMARY

Twenty-eight honey samples were analysed for catalase activity by a spectrophotometric method. Results were compared with previous data on diastase and peroxide accumulation in the same samples. Statistical analysis indicated a direct relation between amounts of catalase and diastase, and an inverse relation between catalase and peroxide accumulation.

INTRODUCTION

Since the time of Auzinger (1910*a*, 1910*b*) there have been reports of the presence of a catalase in honey. These reports (reviewed by Schepartz, 1966) have been open to question owing to the lack of validity of the methods employed. Recently, this problem has been re-examined and conclusive evidence obtained to show that a catalase is indeed present in honey (Schepartz, 1966).

In the latter study, one of the methods used to observe catalatic activity involved following the disappearance of the substrate, hydrogen peroxide, by measurement in an assay system containing peroxidase and *o*-dianisidine. We have taken this method and, with slight modification, applied it to the analysis of a series of honey samples of various types for which analytical data on other enzymes were already available. We were particularly interested in comparing catalase activity with glucose oxidase activity in the same honeys, since these enzymes offset one another in the peroxide equilibrium of honey. The method used for analysis, the data collected and their statistical significance are presented here.

MATERIALS AND METHODS

Honey samples were from those previously listed in a study by White and Subers (1963). All were unheated. Samples without letter prefixes or with M-numbers were stored frozen (−15°C.) prior to use; those with HS-numbers were stored at 4°C.

Hydrogen peroxide was a 30% solution obtained from J. T. Baker Chemical Company,* Phillipsburg, New Jersey, *o*-dianisidine (3,3′-dimethoxybenzidine) from Eastman Organic Chemicals, Rochester, New York, and peroxidase (horse-radish, Type I) from Sigma Chemical Company, St. Louis, Missouri.

Absorbance measurements were made on a Bausch and Lomb Spectronic 505 Recording Spectrophotometer.

* Mention of trade or company names does not imply endorsement by the Department over others of a similar nature not mentioned.

A hydrogen peroxide working solution was made by dilution of the 30% stock to give a concentration between 8 000 and 10 000 μg./ml. This solution was assayed by direct absorbance measurement at 250 mμ, where $\epsilon_{250} = 19{\cdot}25$; thus concentration (M) = absorbance/19·25 (Beers & Sizer, 1952). It was reassayed at frequent intervals and replaced if below the minimum.

Although direct absorbance measurement was a simple and rapid assay for H_2O_2 in standard solutions, it could not be used with catalase reaction mixtures due to high background absorbance. Consequently, the peroxidase-dianisidine system (White, Subers & Schepartz, 1963) was employed, after calibration against the direct absorbance method. Although somewhat more laborious, this technique is more specific and more sensitive than direct absorbance, thus allowing analyses to be run at lower concentrations of peroxide.

Honey samples were analysed for catalatic activity by two successive steps: (1) honey catalase was allowed to react with an excess of H_2O_2; (2) the amount of H_2O_2 remaining was measured by reaction with peroxidase-dianisidine.

Incubation of catalase reaction mixtures was performed at 37°C., because of the low level of activity of the honey enzymes at the temperatures normally used (0 – 20°C.).

Rapid stirring of reaction mixtures and assay samples was accomplished by means of a vortex-type electric stirrer.

Procedure

Preparation of sample. A 10-g. sample was weighed out (to the nearest 0·1 g.), dissolved with 5 ml. water, transferred to a length of dialysis tubing and dialysed against running tap-water (approximately 22°C.) for 24 hours. The dialysed honey solution was then transferred to a volumetric flask and diluted to 25 ml. with water. (Where limited amounts of sample were available, 3 or 5 g. was taken and, after dialysis, diluted to 10 ml.)

Catalase reaction. The complete reaction system contained 3·0 ml. dialysed honey solution, 0·4 ml. 0·6-M sodium phosphate buffer, *p*H 7·0, 0·05 ml. water, and 0·05 ml. H_2O_2 (containing 400 – 500 μg.) in a 10-ml. test-tube. Blanks were run without honey, without substrate. The reaction was started with the addition of H_2O_2. Tubes were mixed rapidly, then incubated at 37°C. At convenient intervals, generally 5, 10, 15, 20 and 30 min., 0·05 ml. portions of reaction mixture were removed and immediately transferred to the assay system given below. Similar withdrawals were made at at least three of the above intervals from the blank containing peroxide but no honey (peroxide blank). Only one withdrawal (at any convenient time) was necessary from the blank containing honey but no substrate (honey blank), since this blank remained constant.

Peroxide assay system. The portions withdrawn from the reaction mixture above were introduced into the following assay system. Each test-tube (10 ml. capacity) contained: 3·2 ml. 0·2-M sodium phosphate buffer, *p*H 6·1; 0·1 ml. *o*-dianisidine (3·5 mg./ml. in 95% ethanol); 1 drop peroxidase (0·04 mg./ml. in above buffer); 0·15 ml. water. Reaction with the peroxidase quickly removed the H_2O_2 from further action by the catalase. After mixing, the assay tubes were allowed to stand at room temperature for 5 min. to allow complete development of the dianisidine

colour. At the end of this time, 2 drops of concentrated HCl were added and the tubes mixed again. This served to reduce the *p*H to 2 and shift the colour to a maximum absorbance at 402 mμ. After an additional 5 min., but no longer than 15 min., the absorbances were measured at 402 mμ against an assay blank prepared similarly, but containing water instead of catalase reaction mixture. In this assay system the absorbance is linear from 0·2 to 2·8 μg./ml., and the following relationship holds:

$$\text{absorbance} \times 3{\cdot}64 = \mu\text{g. } H_2O_2/\text{ml.}$$

TABLE 1. Evaluation of k from absorbance data

t	*Absorbance*				x			k
(min.)	B_p	B_h	S	S_{corr}	(μg.H_2O_2)[b]	x_o/x	1n x_o/x	(from graph)[c]
0	—	—	0·466[a]	0·466	416(x^o)	—	—	—
5	0·445	0·011	0·469	0·458	408	1·020	0·0198	0·0099
10	0·465	„	0·450	0·439	392	1·061	0·0592	0·0085
15	0·465	„	0·435	0·424	378	1·101	0·0961	0·0080
20	0·470	„	0·410	0·399	356	1.169	0·156	0·0092
33	0·485	„	0·375	0·364	325	1·280	0·247	0·0082
	0.466 average							0·0088 average

t = time; B_p = peroxide blank; B_h = honey blank; S = sample; S_{corr} = sample corrected for blanks; x_o = initial substrate; x = substrate at time t; k = rate constant = $\frac{1}{t} - 1\text{n } x_o/x$.

a Average B_p absorbance is taken as t_o value, since it levels off after first 5 minutes.

b x is total μg.H_2O_2 present as calculated from absorbance ($S_{corr} \cdot 3{\cdot}64 \cdot$ dilution factor = x).

c k is slope of line in Fig. 1.

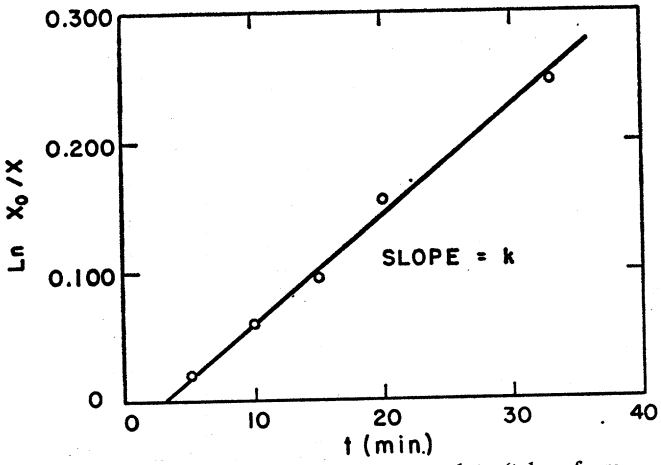

FIG. 1. Plot of first-order reaction rate data (taken from Table 1) for evaluation of k

Calculations

An example of how the data were handled is given in Table 1. Since the decomposition of H_2O_2 by honey catalase is a first-order reaction (Schepartz, 1966), and $k = \frac{1}{t} \ln x_o/x$ where k is the rate constant, t is time, x_o is the initial substrate, and x is the substrate at t), a graph of $\ln x_o/x$ against t gives a straight line up to at least the first 30 minutes (Fig. 1). The slope of this line is k.

Whereas k is a measure of the catalatic activity of a honey, it does not take into account the size of the sample. Hence we have employed the term, K_f (derived from the old expressions for catalase potency): $K_f = kD/W$, where k is the rate constant discussed above (in min.$^{-1}$), D is the dilution factor and W is the weight of honey in grams. Thus K_f is an expression of catalase activity per gram of honey. Using the k value found in Table 1, K_f is then calculated as follows:

$$K_f = kD/W$$
$$= \frac{0{\cdot}0088}{\text{min.}} \times \frac{10}{3} \times \frac{1}{3\text{g.}}$$
$$= 9{\cdot}8 \times 10^{-3}/\text{g. min.}$$

TABLE 2. Comparison of catalase with diastase and peroxide accumulation in honey

No.	*Honey type*	*Diastase*[a]	*Peroxide accumulation*[a]	*Catalase*[b]
5	Alfalfa	18·2	3·0	0
74	Basswood-Clover	30·9	9·4	9·8
104	Summer blend	10·8	1·0	0
153	Buckwheat	31·6	72	9·1
174	Alsike & Sweet clovers	18·2	23	2·2
193	Sweet clover	22·6	6·6	0·5
200	Sweet clover	10·2	5·0	0
331	Gallberry	21·4	38	0
349	Heartsease	35·3	15	11·6
355	Horsemint	21·7	252	4·3
369	Mesquite	8·3	9·0	0·5
372	Mint	15·0	0·0	0
383	Orange-Grapefruit	8·7	35	0
384	Orange-Grapefruit	11·5	50	10·7
410	Raspberry	14·0	15	0
424	Sourwood	15·6	18·5	0
431	Spanish-needle—Heartsease	43·5	175	7·1
453	Tulip tree	18·5	33	8·7
471	Vetch	21·1	170	6·0
HS33	Fall blend	30·6	120	16·3
HS35	Fall blend	45·8	100	14·0
HS36	Clover	15·1	13·3	1·8
HS37	Fall blend	46·2	83	17·8
HS38	Cotton	16·8[c]	310	7·5
M266	Cotton	16·8[c]	360	4·4
M267	Cotton	12·1[c]	320	0
M269	Cotton	14·8[c]	245	3·2
M270	Cotton	12·3[c]	325	3·6

[a] For botanical names and explanation of units, see White & Subers (1963).
[b] $K_f \times 10^3$
[c] Not previously reported.

RESULTS AND DISCUSSION

A series of 28 honey samples was analysed for catalatic activity. Selection of samples was based on availability and interest in the glucose oxidase activity as indicated by peroxide accumulation. The results are given in Table 2. For comparison, information on diastase and peroxide accumulation is also shown. These values were originally reported by White & Subers (1963), except as noted.

The data were plotted as illustrated in Figs. 2 and 3. The points show some degree of scatter in Fig. 2, but they appear to be within the indicated band-lines, suggesting a direct relation between catalase and diastase. A statistical evaluation corroborated this observation ($F = 34{\cdot}82$, $r = +0{\cdot}757$)*, showing a highly significant and direct correlation between the two enzymes. In Fig. 3, the points fall into two groups: (1) where both catalase and peroxide accumulation are very low, and (2) where at least one is at a moderate or higher level. If we neglect the first group as being virtually devoid of catalase and glucose oxidase, the second group falls within a band having a negative slope and, analysed statistically, shows a highly significant *inverse* correlation ($F = 14{\cdot}01$, $r = -0{\cdot}707$)‡ between catalase and peroxide accumulation.

Since diastase and catalase are both enzymes and subject to the same environmental conditions, we would expect honeys with considerable diastase to be fairly high in catalase also. The data bear this out. That catalase and peroxide accumulation should be inversely related is quite logical, since the peroxide level in a

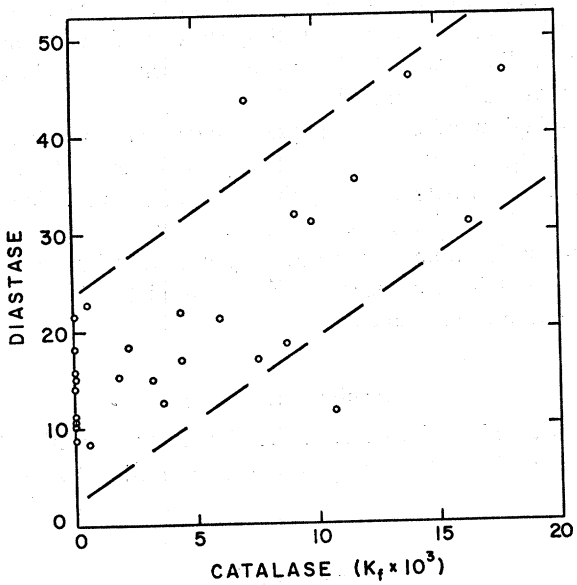

FIG. 2. Relation between catalase and diastase activities in honey samples (from Table 2)

*A value of F of 7·72 or greater indicates that the relationship is highly significant (1% level of significance); r is the correlation coefficient.

‡ A value of F of 8·86 or greater indicates that the relationship is highly significant (1% level of significance).

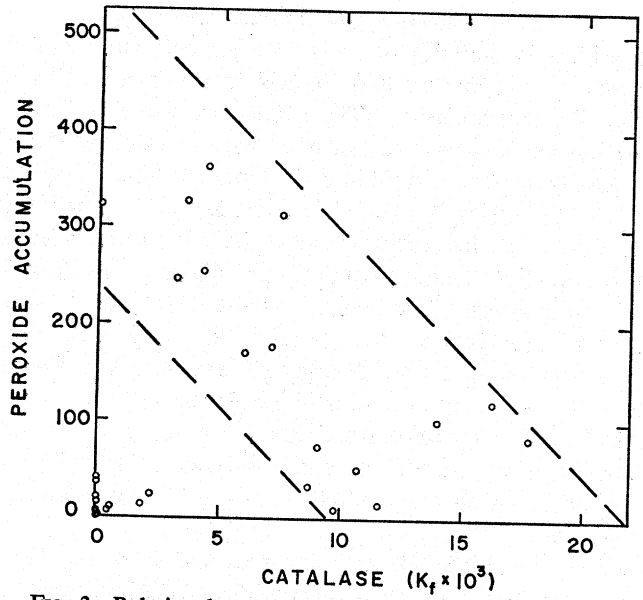

FIG. 3. Relation between catalase and peroxide accumulation in honey samples (from Table 2)

honey will be determined in part by the amount of catalase activity. While peroxide is being formed by the action of glucose oxidase on honey glucose, the catalase destroys it. Thus with both enzymes present, a tendency toward a peroxide equilibrium would be expected.

From the limited number of analyses made here, we conclude that those honeys possessing significant diastase activity will generally contain significant catalase activity. Also, where a honey has significant peroxide accumulation, it will probably be low in catalase, and visa versa. It must be remembered, however, that a honey with considerable catalase activity may also have a significant amount of glucose oxidase, but its presence will be obscured by the fact that, due to the catalase, little peroxide accumulates.

ACKNOWLEDGEMENT

The authors are indebted to Dr. J. W. White, Jr., for his continued interest and encouragement and to Mr. J. N. Boyd and Miss M. J. Smithey for the statistical analyses.

REFERENCES

AUZINGER, A. (1910*a*) Ueber Fermente im Honig und dem Wert ihres Nachweises für die Honigbeurteilung. *Z. Unters. Nahr.-u. Genussmittel* 19 : 65–83

——— (1910*b*) Weitere Beiträge zur Kenntnis der Fermentreaktionen des Honigs. *Z. Unters. Nahr.-u. Genussmittel* 19 : 353–362

BEERS, R. F., JR. & SIZER, I. W. (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. biol. Chem.* 195 : 133–140

SCHEPARTZ, A. I. (1966) Honey catalase: occurrence and some kinetic properties. *In press*

WHITE, J. W., JR. & SUBERS, M. H. (1963) Studies on honey inhibine. 2. A chemical assay. *J. apic. Res.* 2(2) : 93–100

WHITE, J. W., JR., SUBERS, M. H. & SCHEPARTZ, A. I. (1963) The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. *Biochim. biophys. Acta* 73 : 57–70

BEE RESEARCH ASSOCIATION

The Bee Research Association is the central clearing house for information about research on bees and related subjects, including beekeeping and covering all species of bees.

The Association publishes three journals:

Journal of Apicultural Research, an international journal printed in English, for original research papers.

Apicultural Abstracts, a quarterly survey of scientific papers from world literature, in English.

Bee World, the official organ of the Association, which provides beekeeper and scientist with news and articles on recent developments.

Members are entitled to receive one or more of these journals. Membership has other advantages, including use of the Association's extensive Library of books, journals, reprints, translations and pictures, which are lent by post to any country in the world.

The journals can also be obtained by subscription.

Further details, including a complete list of B.R.A. publications, from:

BEE RESEARCH ASSOCIATION

Woodside House, Chalfont Heights, Gerrards Cross, Bucks., England